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(57) Abstract

Peptide reagents and compositions thereof which reduce blood clotting initiated by the ternary complex of tissue factor (TF), FVIIa and FXa. The peptides have the amino acid sequences Thr-Leu-Tyr-Tyr-Trp-Arg-Ala-Ser-Ser-Thr, Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr and Gly-Gly-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr.

PEPTIDE FRAGMENTS OF TISSUE FACTOR AND THEIR USE FOR TREATMENT AND PREVENTION OF CLOTTING DISORDERS

The present invention is concerned with peptide reagents and compositions thereof which reduce blood clotting initiated by the ternary complex of tissue factor (TF), FVIIa and FXa. Reducing the formation of FX coagulant activity (FXa) by interfering with the formation of the ternary complex TF/FVIIa/FXa inhibits TF-dependent blood clotting. Thus the extrinsic pathway of coagulation is inhibited.

Blood clotting relies upon a cascade of enzymatic reactions which eventually results in the formation of a fibrin clot (Furie B. & Furie B.C.: Molecular and cellular biology of blood coagulation. N. Eng. J. Med. 326, 800, 1992). The triggering mechanism is either initiated by contact of blood with an artificial surface (intrinsic coagulation pathway) or by TF at a vessel wall lesion (extrinsic coagulation pathway). The process of blood coagulation is a defence mechanism which prevents blood loss following vessel wall injury (hemostasis). However, a similar process may also be triggered at a vascular lesion where blood loss is not a threat, but which unfortunately may result in thrombus formation (thrombogenesis). It is thought that the extrinsic pathway of coagulation is the dominating one in vivo, both in hemostasis and thrombosis (Gailani D. & Broze G.J.: Factor IX activation in a revised model of blood coagulation. Science 253, 909, 1991; and Nemerson Y.: The tissue factor pathway of coagulation. Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition. Eds. R.W. Colman, J. Hirsh, V.J. Marder & E.W. Salzman. J.B. Lippincott Company, Philadelphia, p81, 1994).

FX is activated by the catalytic activity of the binary TF/FVIIa complex in the presence of Ca^{2+} on

phospholipid-rich surfaces (Nemerson Y.: Tissue Factor and Hemostasis. Blood 71, 1, 1988). During this event, FX complexes with the preformed TF/FVIIa complex and is subsequently activated and released. Thus, a ternary complex is formed which has FX coagulant activity (FXa). Activation of this extrinsic pathway of coagulation leads to fibrin formation which apparently is of prime importance in development of arteriosclerotic lesions and in reocclusion and restenosis following endarterectomy (Weiss H.J., Turitto V.T., Baumgartner H.R., Nemerson Y. & Hoffmann T.: Evidence for the presence of tissue factor activity on subendothelium. Blood 73, 968, 1989; Hultin M.S.: Fibrinogen and factor VII as risk factors in vascular disease. Progress in Hemostasis and Thrombosis. Volume 10, Ed. B.S. Coller, W.B. Saunders, Philadelphia, p215, 1991; and Jang I.K., Gold H.K., Leinbach R.C., Fallon J.T, Collen D. & Wilcox J.N.: Antithrombotic effect of a monoclonal antibody against tissue factor in a rabbit model of platelet-mediated arterial thrombosis. Arterioscler. Thromb. 12, 948, 1992). Relatively little is known about the molecular interactions between TF and FVIIa and between TF/FVIIa and FX.

Mouse TF, in contrast to rabbit TF, does not support the procoagulant activity of human FVIIa on FX (Andrews B.S., Rehemtulla A., Fowler B.J., Edgington T.S. & Mackman N.: Conservation of tissue factor sequence among three mammalian species. Gene 98, 265, 1991). Whether this effect is due to lack of compatibility between mouse TF and human FVII and/or FX is not known. Recently, three putative binding sites for FVIIa and one for FX were identified in human TF (Harlos K., Martin D.M.A., O'Brien, Jones E.Y., Stuart D.I., Polikarpov I., Miller A., Tuddenham E.G.O. & Boys C.W.G.: Crystal structure of the extracellular region of human tissue factor. Nature 370, 662, 1994). When studying the species homology for these binding regions,

we found striking homology for the putative FVIIa binding sites (>80% for 10 residues comprising putative FVIIa binding sites). However, for the FX recognition site there is low homology between man and mouse (40 % for 10 residues, residues are identified below), whereas the homology between man and rabbit is higher (70 % for 10 residues, residues are identified below). It therefore seemed likely that the incompatability between mouse TF and human FVIIa and/or FX is localized to this region, resulting in impaired FX binding and FX activation. The putative FX binding site on the TF molecule is outlined below:

FX Putative Binding Site

Rabbit:	152 - Thr-Leu-Tyr-Tyr-Trp-Arg-Ala-Ser-Ser-Thr - 161 (70%)
Man:	154 - Thr-Leu-Tyr-Tyr-Trp-Lys-Ser-Ser-Ser-Ser - 163
Mouse:	158 - Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr - 167 (40%)

Site-directed mutagenesis of TF as well as synthetic TF peptides has in addition indicated a domain of the TF molecule which appears important for the activation of FX by the binary TF/FVIIa complex (Edgington T.S. & Morrissey J.H., U.S. Patent Number 5,110,730, May 5, 1992). This domain is located at residues 152-169 of the mature TF molecule.

We have surprisingly found that peptides of smaller size, and not based on the primary sequence of human TF, are very potent inhibitors of TF-dependent coagulation in humans.

The invention thus provides peptides having the amino acid sequences Thr-Leu-Tyr-Tyr-Trp-Arg-Ala-Ser-

Ser-Thr, Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr and Gly-Gly-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr and functional equivalents thereof wherein one or more of the amino acids in the above stated sequence may be modified or absent, all amino acids other than glycine being in the conventional L form.

The invention also provides the abovementioned peptides for use in the treatment or prevention of blood clotting disorders or problems in a human subject.

Conservative amino acid substitutions are well known in the art, for example Ser for Thr and vice versa, Lys for Arg and vice versa, and replacement of a neutral amino acid such as Gly, Ala, Leu or Ile with another amino acid from this group. Such substitutions are included in the term "modified" as used herein.

Additional N- or -C terminal amino acids may be present, for example extending along the known sequence of rabbit or murine TF. Thus for example murine peptides may comprises Y or GY at the N-terminal end or some or all of GKKTNI at the -C terminal end.

The peptides may also be in cyclised form, for example by formation of an amide bond between the N- and -C termini or between one of these termini and an appropriate side chain in one of the amino acids in the sequence. If the peptide contains cysteine residues, disulphide bridges may be present.

The total number of amino acids in the peptide may range from 3 to 18, preferably from 8 to 18.

The peptides of the invention differ from those of previous publications in this particular area and from the above cited patent, since they are not based on the

primary sequence of human TF. In contrast, they have primary sequences similar to murine and rabbit TF, showing between 40% and 70% homology respectively with human TF. Furthermore, kinetic analysis reveals that both peptides inhibit extrinsic coagulation by competing with the binary complex of TF/FVIIa for FX. This implies that the conversion of FX to FXa by the TF/FVIIa complex is inhibited. Thus, the peptides have unique amino acid sequences, inhibitory capacity in a relevant bioassay and a well established mechanism of inhibition. Such inhibitors represent a novel approach for inhibition of coagulation and thrombus formation in humans. As such, these peptide and compositions thereof may be used as human antithrombotics, both in prophylaxis and treatment.

The activity of the peptides Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr and Gly-Gly-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr and functional equivalents thereof is particularly surprising given that native mouse TF does not support the human procoagulant activity of human FVIIa on FX (see the reference cited above).

The inhibitory capacity of the peptides was measured by a so-called lipidated TF assay which is a chromogenic assay measuring the FXa generated by the binary complex of TF/FVIIa. FVIIa (5 pM final concentration) and FX (20 nM) were combined in the presence of different concentrations of peptide and incubated for 15 min at ambient temperature. TF (5 pM) and $CaCl_2$ (5mM) were added to initiate the reaction. Reactions were quenched with EDTA and the FXa activity was measured in an amidolytic assay, using the chromogenic FXa substrate S2765 (trade name). Data from these studies with this assay are shown in Figure 1, and Table 1 hereinafter gives the corresponding IC values. Data are plotted as the percentage of the rate of FXa

formation determined without peptide versus the concentration of the following peptides: Hu#154-163 (■), mu#158-167 (•), mu#160-167 + GG (▲) and rb#152-161 (♦). It is noted that the IC values for mu#158-167, mu#160-167, mu#160-167 + GG and rb#152-161 are in the very low μM range.

Table 1: **INHIBITION OF TF-DEPENDENT FX ACTIVATION BY TF PEPTIDES FROM MAN, RABBIT AND MOUSE**

Residues	Peptide sequence	Number of Residues	Homology to Human sequence (%)	IC50* (uM)
Hu#152-169	IYTLYYWKSSSSGKKTAK	18		68
Hu#154-167	TLYYWKSSSSGKKT	14		157
Hu#154-163	TLYYWKSSSS	10		220
Hu#156-160	YYWKS	5		330
Rb#152-161	TLYYWRASST	10	70	17
Mu#156-173	GYIITYRKGSSTGKKTNI	18	50	150
Mu#158-167	IITYRKGSST	10	40	33
Mu#158-167 scrambled	YSGITSKTIR	10	0	no inhibition
Mu#159-167	ITYRKGSST	9	44	86
Mu#160-167	TYRKGSST	8	50	30
Mu#160-167 + GG	GGTYRKGSST	10	40	17

* The concentration of peptide inhibiting the rate of FX activation by 50%. Values are based on at least triplicate separate determinations

The inhibitory mechanism of FXa formation was elucidated by analysis of Dixon plots as shown in Figures 2A, 2B and 3A. Different concentrations of peptide were mixed with FVIIa and three different concentrations of FX and, after addition of TF, the rate of FXa formation was determined. In another experiment, different concentrations of peptide were mixed with FVIIa and three different concentrations of TF, in excess of FVIIa. After addition of FX, the rate of FXa formation was determined. Results were plotted according to Dixon, the inverse of the rate of FXa formation versus peptide concentration. For different FX concentrations peptides mu#158-167 and mu#160-167, produced linear regression lines, which intercepted on the abscissa. For different TF concentration mu#158-167 produced linear regression lines which intercepted above the abscissa. These results are consistent with a mechanism where the peptide mu#158-167 competes with TF or FVIIa/TF complex for interaction with FX.

Further investigation into the inhibitory mechanism of FXa formation was accomplished by analysis of the Lineweaver-Burk plot shown in Figure 3B. Different concentration of the peptide mu#160-167 (between 2 and 100 μM) were mixed with various concentrations of FX (between 5 and 50 nM). Results were plotted according to Lineweaver-Burk, the inverse of the rate of FXa formation versus the inverse of FX concentration. The peptide mu#160-167 produced linear regression lines which intercepted on the abscissa indicating a purely non-competitive mode of inhibition. The inset in Figure 3B is a re-plot of slope (■) and intercept (•) from the Lineweaver-Burk plot versus concentration of peptide and again indicates non-competitive inhibition.

The present invention also provides pharmaceutical compositions containing one or more of the peptides of the invention or salts thereof.

Salts of the peptides include physiologically acceptable acid addition salts such as the hydrochloride.

The compositions according to the invention may be presented, for example, in a form suitable for nasal or parenteral administration.

Thus the compounds according to the invention may be presented in the conventional pharmacological forms of administration, such as nasal sprays, solutions and emulsions. Conventional pharmaceutical excipients as well as the usual methods of production may be employed for the preparation of these forms. Organ specific carrier systems may also be used.

Injection solutions may, for example, be produced in the conventional manner, such as by the addition of preservation agents, such as p-hydroxybenzoates, or stabilizers, such as EDTA. The solutions are then filled into injection vials or ampoules.

Nasal sprays may be formulated similarly in aqueous solution and packed into spray containers either with an aerosol propellant or provided with means for manual compression. Capsules containing one or several active ingredients may be produced, for example, by mixing the active ingredients with inert carriers, such as lactose or sorbitol, and filling the mixture into gelatin capsules.

Dosage units containing the compounds of this invention preferably contain 0.1-10mg, for example 1-5mg of the peptide or salt thereof.

As indicated above, one aspect of the invention provides peptides according to the invention for use in the treatment or prevention of blood clotting disorders

or problems in a human subject. Blood clotting disorders include thrombosis (particularly vascular thrombosis or deep vein thrombosis), acute myocardial infarction, restenosis, reclosure, angina, cerebrovascular disease, peripheral arterial occlusive disease, hypercoagulability, DIC and pulmonary embolism. The peptides according to the invention can also be used to prevent occurrence of blood clotting problems caused by, for example, injury to blood vessels during thrombolytic therapy, grafting surgery, vessel patency restoration etc. Blood clotting disorders may be triggered by sepsis due to production of TNF-α or IL-1.

In a still further aspect, the present invention also provides a method of treatment of blood clotting disorders in the human body, said method comprising administering to said body one or more peptides according to the invention or salts thereof. Prophylactic methods of treatment are also provided, whereby a peptide according to the invention is administered to a patient to prevent or reduce the occurrence of possible blood clotting problems, for example during surgery or other invasive techniques. The peptide will of course normally be administered in the form of a pharmaceutically acceptable composition.

In another aspect, the present invention provides a process for the preparation of the peptides according to the invention.

The peptides of the present invention can be prepared by methods known in the art. Typically, the desired sequences are assembled by solid-phase peptide synthesis. Standard procedures for the synthesis strategy employed for the examples of this invention are described in E. Atherton & R.C. Sheppard, 'Solid phase peptide synthesis: a practical approach, 1989, IRL Press, Oxford. For example, a synthesis resin with an

acid-labile linker group, to which the desired protected C-terminal amino acid residue has been esterified, is used. In the following examples, so-called TentaGel resins with a trityl-derived linker were applied (Bayer, E., Clausen, N., Goldammer, C., Henkel, B., Rapp, W. & Zhang, L. (1994) in *Peptides: Chemistry, Structure and Biology* (Hodges, R.S. & Smith, J.A., eds.), pp. 156-158, ESCOM, Leiden). The amino-protecting group is then removed and the second amino acid in the sequence is coupled using a suitable condensation reagent. Amino acids with semi-permanent amino protecting groups and permanent protecting groups for the functional side chains are employed. Amino-deprotection and coupling cycles are then repeated in alternating steps until the sequence of interest is assembled. Finally the permanent side-chain protecting groups are removed and the peptide is cleaved from the synthesis resin, usually simultaneously through treatment with a suitable acidic reagent.

Alternatively, the peptides can be synthesised through solution peptide synthesis methods known in the art, either in a step-wise manner from the carboxyl terminus and/or through the application of segment condensation or ligation methods, employing comprehensive or minimal protection strategies. Combined solution-solid phase segment condensation approaches can also be applied.

Generally, the reactive groups present (for example amino, hydroxyl, thiol and carboxyl groups) will be protected during overall synthesis as indicated above. The final step in the synthesis will thus be the deprotection of a protected derivative of the peptides of the invention. A wide choice of protecting groups for amino acids is known (see, *e.g.*, Greene, T.W. & Wuts, P.G.M. (1991) *Protective groups in organic synthesis*, John Wiley & Sons, New York). Thus for

example amino protecting groups which may be employed include 9-fluorenylmethoxycarbonyl (Fmoc), benzyloxycarbonyl, t-butyloxycarbonyl, etc. It will be appreciated that when the peptide is built up from the C terminal end, an amino-protecting group will be present on the α-amino group of each new residue added and will need to be removed selectively prior to the next coupling step. One particularly useful group for such temporary amine protection is the Fmoc group which can be removed selectively by treatment with piperidine in an organic solvent. Carboxyl protecting groups which may for example be employed include readily cleaved ester groups such as t-butyl and benzyl, as well as esters with solid phase-bound linkers, e.g. p-alkoxybenzyl, trityl, etc. It will be appreciated that a wide range of other such groups are known in the art. The use of all such protecting groups and the processes described above falls within the scope of the present invention.

The invention is illustrated by the following Examples.

Example 1

H-Thr-Leu-Tyr-Tyr-Trp-Lys-Ser-Ser-Ser-Ser-OH (huTF residues 154-163)

The peptidyl resin corresponding to the above sequence was assembled on Fmoc-Ser(Bu^t)-[TentaGel S Trt resin] (0.2 mmol/g; from Rapp Polymere GmbH, Tübingen, Germany) using an Applied Biosystems model 433A peptide synthesizer. Fmoc deprotection was achieved with conductivity monitoring using 20% piperidine in N-methylpyrrolidone (NMP). The washing solvent was NMP. The residues (from the carboxyl terminus) were assembled using double couplings with 10-fold molar excess of Fmoc-amino acids and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt)/Pr^i_2NEt in NMP using 75 min coupling cycles. Prior to Fmoc-deprotection at each sequence position capping was carried out using a solution of acetic anhydride (4.7 % v/v)/Pr^i_2NEt (2.2% v/v)/HOBt (0.2 % w/v) in N,N-dimethylformamide. The amino acid-side chain protecting groups used were t-butoxycarbonyl for Lys and t-butyl for Ser, Thr and Tyr. The final Fmoc-deprotected and washed (dichloromethane) peptidyl resin was dried *in vacuo*. An aliqout (300 mg) was treated with a mixture containing phenol, 1,2-ethanedithiol, thioanisole, water and CF_3COOH (0.75:0.25:0.5:0.5:10, w/v/v/v/v) for 3h. The resin residue was then filtered off and washed with small quantities of neat CF_3COOH. The combined filtrate and washings were triturated with Et_2O to obtain the crude peptide. The precipitate was collected by filtration, washed with Et_2O and then taken up in 0.1 % aq CF_3COOH and lyophilised. An aliquot (25 mg) of the crude product was redissolved in 0.1 % aq CF_3COOH (3 mL), filtered and purified by preparative RP-HPLC. The column (Vydac 218TP1022, 2.2 x 25 cm) was eluted at 10

ml/min with a gradient of 0 to 20% MeCN in 0.1 % aq CF_3COOH over 90 min. Appropriate peak fractions were pooled and lyophilised to afford 12 mg of pure peptide. Analytical RP-HPLC: t_R = 19.6 min, purity 99% (Vydac 218TP54, 0.46 x 25 cm, 0-30 % MeCN in 0.1 % aq CF_3COOH over 20 min at 1 ml/min, λ = 215 nm). FAB-MS: $[M + H]^+$ = 1221.6 m/z, $C_{57}H_{81}N_{12}O_{18}$ = 1221.3. Amino acid analysis: Leu 1.01(1), Lys 1.05(1), Ser 3.96(4), Thr 1.00(1), Tyr 1.98(2), Trp was not determined.

Example 2

H-Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr-OH (muTF residues 158-167)

The peptidyl resin corresponding to the above sequence was assembled on Fmoc-Thr(Bu^t)-[TentaGel R Trt resin] (0.17 mmol/g; from Rapp Polymere GmbH, Tübingen, Germany) in a similar fashion to the corresponding peptidyl resin in Example 1. The side chain protecting group used for Arg was 2,2,5,7,8-pentamethylchroman-6-sulphonyl. The completed peptidyl resin was worked up and an aliquot (500 mg) treated with the same acidolysis reagent as in Example 1. After precipitation from Et_2O, the product was dissolved in glacial AcOH and lyophilised. The crude product (99 mg) was redissolved in 10% aq MeCN containing 0.1 % CF_3COOH (6 mL), filtered and purified in two batches by preparative RP-HPLC using the same conditions as described in Example 1. Appropriate peak fractions were pooled and lyophilised to afford 74 mg of pure peptide. Analytical RP-HPLC: t_R = 14.7 min, purity 98 % (Vydac 218TP54, 0.46 x 25 cm, 0-30% MeCN in 0.1% aq CF_3COOH over 20 min at 1 ml/min, λ = 215 nm). FAB-MS $[M + H]^+$ = 1125.5 m/z, $C_{49}H_{84}N_{14}O_{16}$ 1125.3. Amino acid analysis: Arg 1.02(1), Gly 1.02(1), Ile 1.22(2), Lys 1.00(1), Ser 2.01(2), Thr 1.95(2), Tyr

1.01(1).

Example 3

H-Thr-Leu-Tyr-Tyr-Trp-Arg-Ala-Ser-Ser-Thr-OH (rbTF residues 152-161)

The peptidyl resin corresponding to the above sequence was assembled on Fmoc-Thr(Bu^t)-[TentaGel R Trt resin] (0.17 mmol/g; from Rapp Polymere GmbH, Tübingen, Germany) in a similar fashion to the corresponding peptidyl resin of Example 1. The side-chain protecting group used for Arg was 2,2,5,7,8-pentamethylchroman-6-sulphonyl. The completed peptidyl resin was worked up and an aliquot (520 mg dry weight) treated with the same acidolysis reagent as in Example 1. After precipitation from Et_2O, the product was dissolved in glacial AcOH and lyophilised. An aliquot of the crude product (40 mg) was redissolved in 10% aq MeCN containing 0.1% CF_3COOH (4 mL), filtered and purified by preparative RP-HPLC using the same conditions as described in Example 1. Appropriate peak fractions were pooled and lyophilised to afford 25 mg of pure peptide. Analytical RP-HPLC: t_R = 20.6 min, purity 97 % (Vydac 218TP54, 0.46 x 25 cm, 0-30% MeCN in 0.1 % aq CF_3COOH over 20 min at 1 ml/min, λ = 215 nm). FAB-MS: $[M + H]^+ = 1247.7$ m/z, $C_{58}H_{82}N_{14}O_{17}$ = 1247.4. Amino acid analysis: Ala 1.01(1), Arg 1.02(1), Leu 1.00(1), Ser 1.99(2), Thr 1.97(2), Tyr 2.00(2), Trp was not determined.

The following peptides were made by analogous methods:

Example 4

H-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr-OH (Mu residues 159-167)

Analytical RP-HPLC: t_R = 17.9 min, purity 98% (Vydac 218TP54, 0.46 x 25 cm, 0-15% MeCN in 0.1 % aq CF_3COOH over 20 min at 1 ml/min, λ = 215 nm). MALDI-TOF MS: $[M+H]^+$ = 1013.1 m/z, $C_{43}H_{73}N_{13}O_{15}$ 1012.1. Amino acid analysis: Arg 1.00(1), Gly 1.00(1), Ile 1.01(1), Lys 1.01(1), Ser 2.02(2), Thr 1.96(2), Tyr 1.01(1).

Example 5

H-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr-OH (Mu residues 160-167)

Analytical RP-HPLC: t_R = 15.2 min, purity 99% (Vydac 218TP54, 0.46 x 25 cm, 0-10% MeCN in 0.1 % aq CF_3COOH over 20 min at 1 ml/min, λ = 215 nm). MALDI-TOF MS: $[M+H]^+$ = 900.8 m/z, $C_{37}H_{62}N_{12}O_{14}$ 899.0. Amino acid analysis: Arg 1.00(1), Gly 1.00(1), Lys 1.01(1), Ser 2.01(2), Thr 1.97(2), Tyr 1.00(1).

Example 6

H-Gly-Gly-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr-OH (Mu residues 160-167 + GG)

Analytical RP-HPLC: t_R = 14.6 min, purity 95.6% (Vydac 218TP54, 0.46 x 25 cm, 0-12% MeCN in 0.1 % aq CF_3COOH over 20 min at 1 ml/min, λ = 215 nm). MALDI-TOF MS: $[M+H]^+$ = 1014.3 m/z, $C_{41}H_{68}N_{14}O_{16}$ 1013.0. Amino acid analysis: Arg 0.96(1), Gly 2.99(3), Lys 1.00(1), Ser 2.03(2), Thr 2.01(2), Tyr 0.99(1).

Claims

1. The peptides comprising the amino acid sequences Thr-Leu-Tyr-Tyr-Trp-Arg-Ala-Ser-Ser-Thr, Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr and Gly-Gly-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr and functional equivalents thereof wherein one or more of the amino acids in the above stated sequence may be modified or absent.

2. A peptide as claimed in claim 1 comprising the core amino acid sequence Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr.

3. A peptide as claimed in claim 2 comprising one or more additional N- and/or -C terminal amino acid residues.

4. A peptide as claimed in claim 3 which is Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr, Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr or Gly-Gly-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr.

5. A peptide which is Ile-Ile-Thr-Tyr-Arg-Lys-Gly-Ser-Ser-Thr or Thr-Leu-Tyr-Tyr-Trp-Arg-Ala-Ser-Ser-Thr.

6. A peptide as claimed in any of claims 1 to 5 wherein said peptide is a synthetic peptide.

7. A peptide as claimed in any of claims 1 to 5 for use in the treatment or prevention of blood clotting disorders or problems in a human subject.

8. A pharmaceutical compostion containing a peptide according to any of claims 1 to 5 or a salt thereof.

9. A method of treatment or prevention of blood clotting disorders or problems in a human body, said

method comprising administering to said body a peptide as claimed in any of claims 1 to 5 or a salt thereof.

10. A method of inhibiting or interfering with the binding of tissue factor-Factor VIIa complex to Factor X in a human subject, said method comprising administering to said subject a peptide as claimed in any of claims 1 to 5 or a salt thereof.

11. Use of a peptide as claimed in any of claims 1 to 5 in the manufacture of a medicament for the treatment of blood clotting disorders or problems in a human body.

12. A process for the preparation of peptides as claimed in claim 6 wherein the amino acid sequences are assembled by solid phase or solution peptide synthesis.

Fig. 1

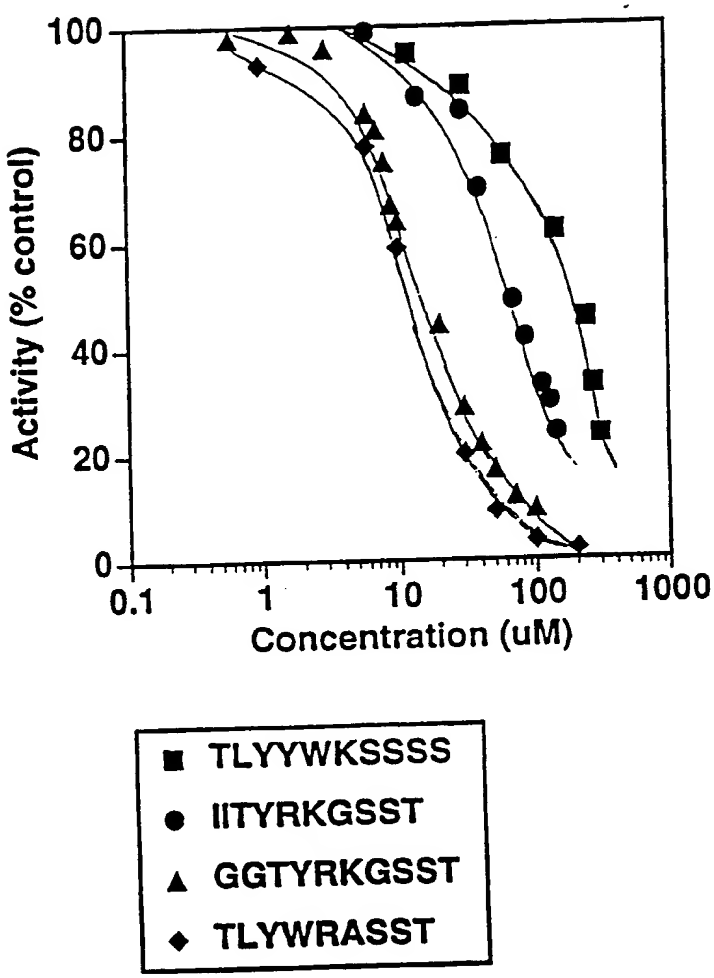

100
80
60
40
20
0
Activity (% control)
0.1
1
10
100
1000
Concentration (uM)
TLYYWKSSSS
IITYRKGSST
GGTYRKGSST
TLYWRASST

Fig 2A.

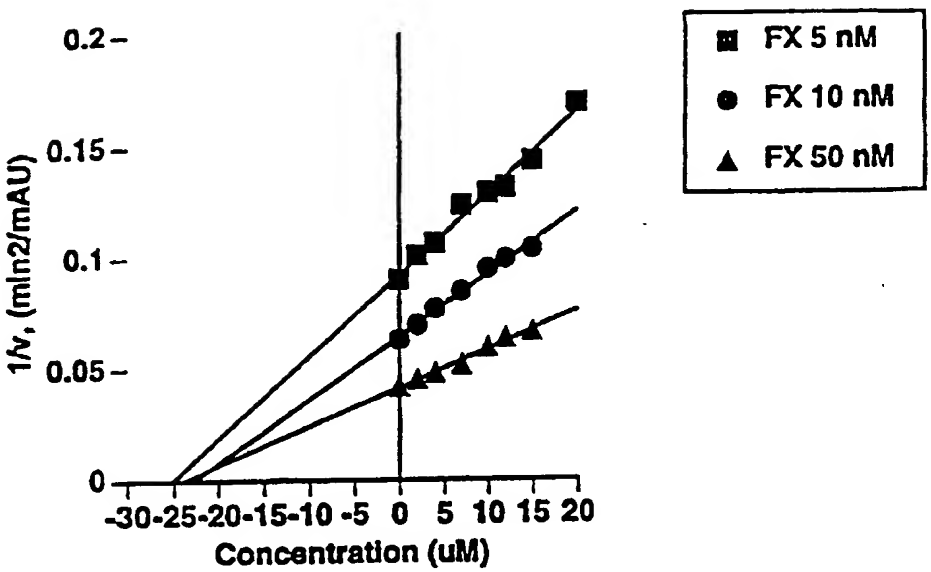

FX 5 nM
FX 10 nM
FX 50 nM
1/v, (min2/mAU)
0.2
0.15
0.1
0.05
0
-30 -25 -20 -15 -10 -5 0 5 10 15 20
Concentration (uM)

Fig. 2B.

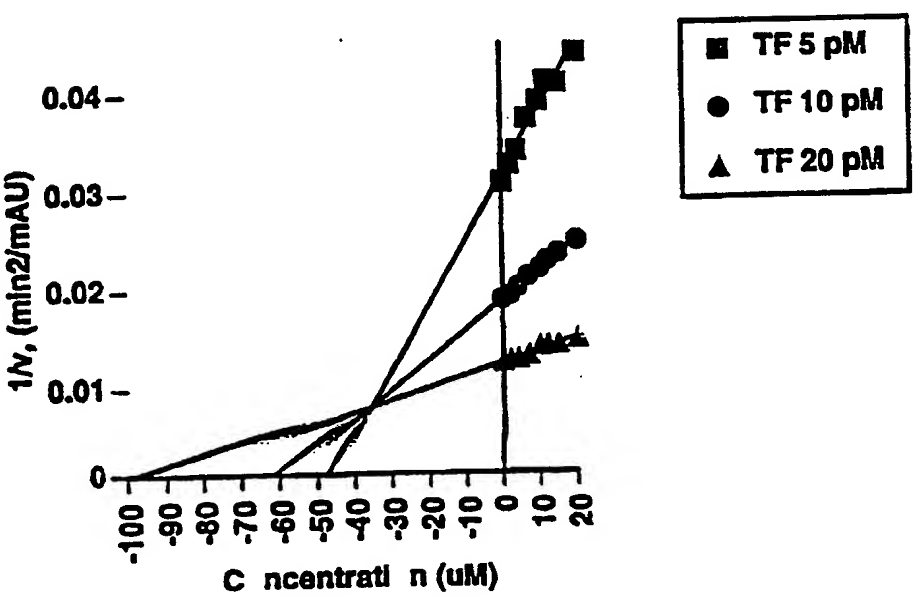
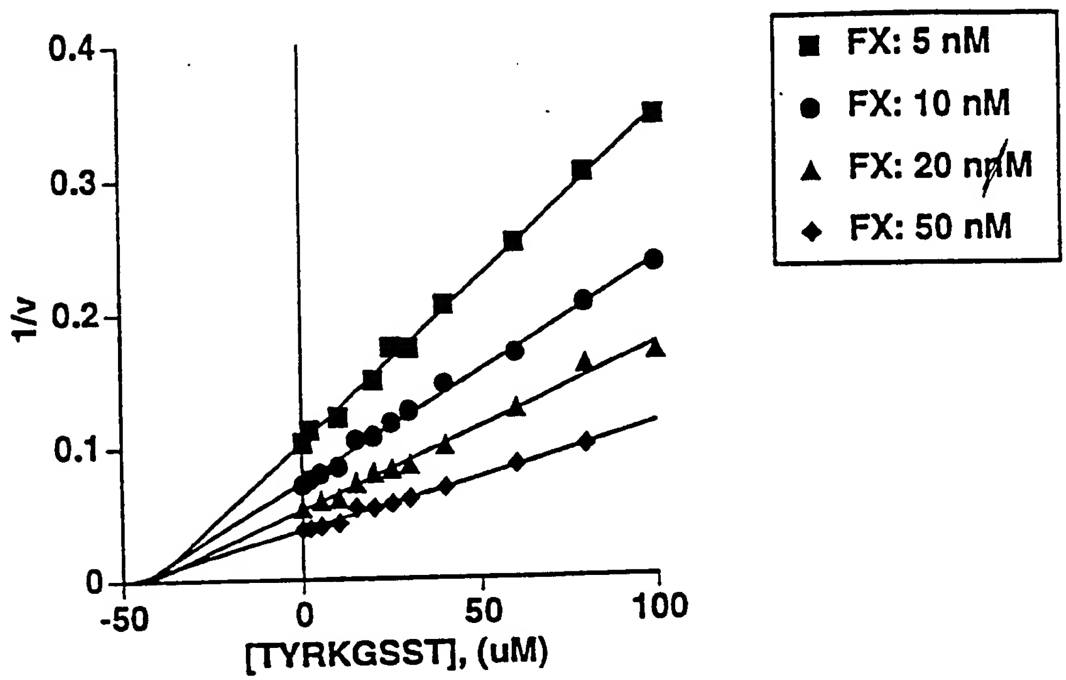
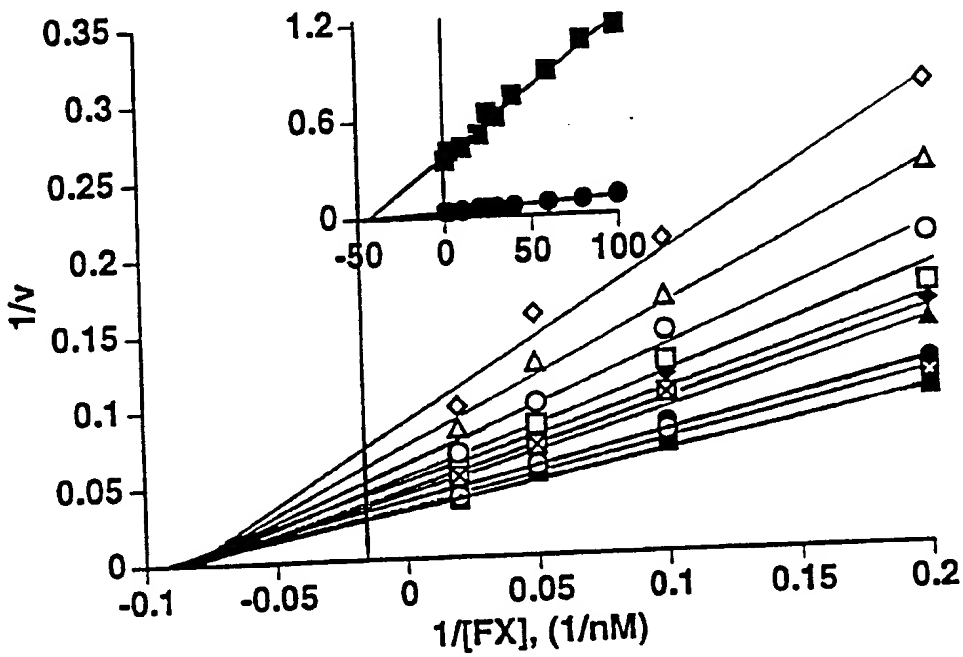

TF 5 pM
TF 10 pM
TF 20 pM
1/v, (min2/mAU)
0.04
0.03
0.02
0.01
0
-100 -90 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20
C ncentrati n (uM)

Fig. 3. A



0.4
0.3
0.2
0.1
0
1/v
-50
0
50
100
[TYRKGSST], (uM)
FX: 5 nM
FX: 10 nM
FX: 20 nM
FX: 50 nM

Fig. 3. ß



1/v
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.1
-0.05
0
0.05
0.1
0.15
0.2
1/[FX], (1/nM)
1.2
0.6
0
-50
0
50
100

INTERNATIONAL SEARCH REPORT

Internatir ' Application No
PCT/GB 96/01363

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/745 A61K38/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 11029 A (SCRIPPS RESEARCH INST ;UNIV PENNSYLVANIA (US); UNIV TEMPLE (US)) 26 May 1994 see page 33, line 30 - page 38, line 21; tables 1,2,4 see page 78, line 10 - page 81, line 16; claims ---	1-11
X	WO 88 07543 A (SCRIPPS CLINIC RES) 6 October 1988 cited in the application see page 32, line 9 - page 37, line 3; claims 21-25,37-18 see page 75 - page 78; tables 1,2,4 --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
19 November 1996	26. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

Internatir ' Application No
PCT/GB 96/01363

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENE (1991), 98(2), 265-9 CODEN: GENED6;ISSN: 0378-1119, 1991, XP002018103 ANDREWS, BRIAN S. ET AL: "Conservation of tissue factor primary sequence among three mammalian species" see figures 2,4 ---	1-11
A	WO 95 00541 A (HOLMES MICHAEL JOHN ;HAFSLUND NYCOMED A S (NO); STEPHENS ROSS WENT) 5 January 1995 see claims; examples -----	1-12

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INTERNATIONAL SEARCH REPORT	International application No. PCT/GB 96/01363

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 9,10
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 9 and 10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

L nation on patent family members

International Application No
PCT/GB 96/01363

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9411029	26-05-94	US-A- 5437864	01-08-95
		AU-A- 5671594	08-06-94
WO-A-8807543	06-10-88	US-A- 5110730	05-05-92
		US-A- 5223427	29-06-93
		AU-B- 605864	24-01-91
		AU-A- 1627488	02-11-88
		EP-A- 0309548	05-04-89
		FI-A- 954347	15-09-95
		GR-A- 88100198	31-01-89
		JP-T- 1503438	22-11-89
		US-A- 5437864	01-08-95
WO-A-9500541	05-01-95	AU-A- 6975594	17-01-95
		CN-A- 1125450	26-06-96
		EP-A- 0703923	03-04-96
		FI-A- 956055	26-01-96
		NO-A- 955067	14-12-95
		ZA-A- 9404337	27-02-95